echinoid in Axonal Outgrowth during *Drosophila melanogaster* embryogenesis

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for graduation with research distinction in Molecular Genetics in the College of Biological Sciences of The Ohio State University

by

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FIGURE LEGEND

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I. Abstract

The Notch signaling pathway (NGS) is a major signal transduction pathway, which regulates the development of the nervous system as well as axonal outgrowth in the embryos of *Drosophila melanogaster*. It is important for cell-cell communication and neuronal precursor cell differentiation. NGS is normally conducted by direct cell-to-cell contact. Signals can be transmitted to neighboring cells where they can activate or suppress a given developmental fate through inter-cellular signal transduction. NGS is also called the Notch cascade because of the series of events that begin extracellularly and end up with changes in gene expressions.

One gene that interacts with the NGS is *echinoid*. It acts as a positive regulator of the Notch pathway where it is thought to be involved in the regulation of the Notch ligand internalization. Notch ligands activate NGS and through a series of catalyzed events the Notch intercellular domain (N^{icd}) and other transcription factors enter the nucleus and regulates various genes, alter gene expression, and recruit proteins to regulate other signals for neurogenesis. This study attempts to answer a few questions about the role of Echinoid (Ed) during neurogenesis. What is the role of Ed in axonal outgrowth? Whether Ed is present in axon formation? If so, where is Ed present? A large portion of this research has been preliminary work in observing Ed. A main focus has been to find the optimal condition for Ed observation. What fixation time for embryos is best for Ed observation in axons *in situ*? What protocol is best used for protein isolation? What dilutions are best for Ed antibodies and HRP-antibodies for western blots? This

study led to unexpected observations of early embryos. Is Ed localized in the nuclei of early syncytial blastoderms? This new observation has led to many new questions for further work on Ed.

II. Introduction

A. *Drosophila melanogaster* embryogenesis

The life cycle of *Drosophila* includes an embryonic stage, where the first part is called the syncytial stage followed by the cellular blastoderm stage (Fig. 1). During the syncytial stage (up to stage 4) the embryo consists of a large cell with many nuclei. There are 9 nuclear divisions before pole cell formation. At stage three the pole cells form at the posterior end, which are the precursors of the germline. At stage four they divide once more and then pinch off, but continue to divide into pole cells (Campos-Ortega and Hartenstein, 1985). Most of the embryo's RNA are maternal at this point and the proteins are translated zygotically. At stage five, cellularization begins to take place and the embryo starts zygotic transcription of RNA. Blastoderm cells move to the periphery and cell membrane formation begins.

Neurogenesis begins at the neuroectodermal layer of stage eight during germ band elongation occurs. First, neuroblasts must be separated from the ectoderm (Campos-Ortega and Hartenstein, 1985). Clusters of cells appear within the neurogenic ectoderm. Within these clusters, cell-cell communication results in limiting potential to a single cell, the neural precursor, which will differentiate into a neuroblast. The neuroblasts organize into characteristic spatial and temporal patterns and divide to become ganglion mother cells (GMC) and neuroblasts. GMCs divide and their cells become the future neurons and glia cells. Stage 11 begins tracheal pit formation, which will give rise to the trachea used for breathing (Campos-Ortega and Hartenstein, 1985). A picture of the mature embryonic central nervous system (CNS) and peripheral nervous system (PNS) is shown in Figure 2.

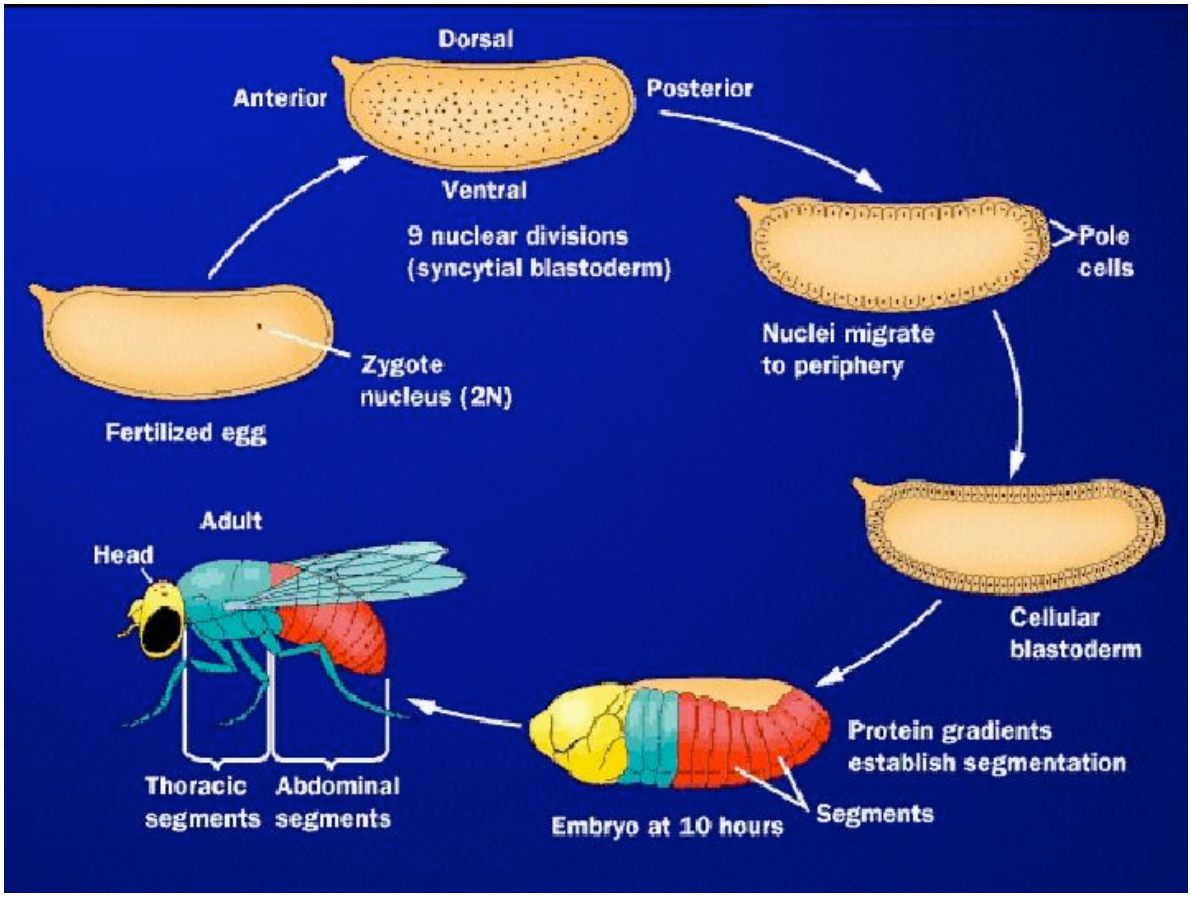


Figure 1 *Drosophila* embryogenesis

Embryonic development of *Drosophila*. (from LIFE: The Science of Biology, Purves et al, 1998).

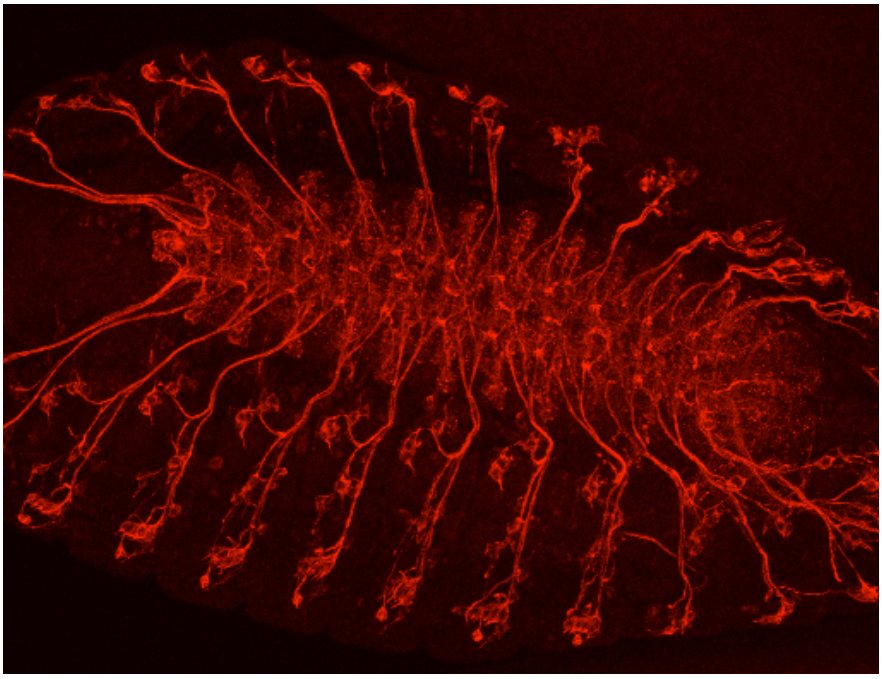


Figure 2 *Drosophila* nervous system

Subset of embryonic nervous system of *Drosophila melanogaster* labeled with monoclonal MAb 22c10.

B. Neurogenesis, Notch signaling pathway (NGS)

During embryonic development of *Drosophila,* neurogenesis (development of the nervous system) requires the function of the NGS, which ultimately limits the neuronal potential in the ectodermal cells (outermost layer) of development. Initiation of neurogenesis in Drosophila requires the selection of an individual neuronal precursor from proneural cluster of ectodermal cells. Within this cluster, one neuroectodermal cell differentiates into a neuroblast while the remaining cells become epidermoblasts (Ahmed et. al. 2003). This differentiation is controlled by cell-to-cell communication mediated by the NGS in a process of lateral inhibition (Fig. 3) (Alberts, 2002). Signals can be transmitted to neighboring cells where they can activate or suppress a given developmental fate through inter-cellular signal transduction.

The Notch receptor is a transmembrane protein where the extracellular domain of the receptor is composed of epidermal growth factor (EGF)-like repeats. The intracellular domain of the Notch protein (N^{icd}) contains an intracellular region that can act as a transcription factor when released upon activation by Notch ligands—Delta or Serrate (Ahmed et al 2003). *echinoid* is a gene that has a critical role in the interaction with the NGS. It acts as a positive regulator of the NGS where it is thought to be involved in the regulation of the Notch ligand internalization (Ahmed et al, 2003). Notch ligands activate the NGS and through a series of catalyzed events that result in the proteolytic cleavage and ultimate release of the N^{icd}, which in turn enters the cell nucleus (Roegers and Jan, 2004).

Interactions between the N^{icd} and other transcription factors regulate various genes, alter gene expression, and recruits proteins to regulate other signals. The Notch

extracellular domain (N^{ecd}) is endocytosed with the Delta ligand into the future neuronal precursor (Fig. 4) (Roegers and Jan, 2004). Notch is a critical pathway for human development. Mutations in Notch pathway genes lead to various pathologies including leukemia, breast cancer, spondylocostal dysostosis, and aortic valve disease (Louvi and Artavanis-Tsakonas 2006). Loss of function mutations of Notch results in a neurogenic phenotype: hyperplasia of neuronal cells in the nervous system.

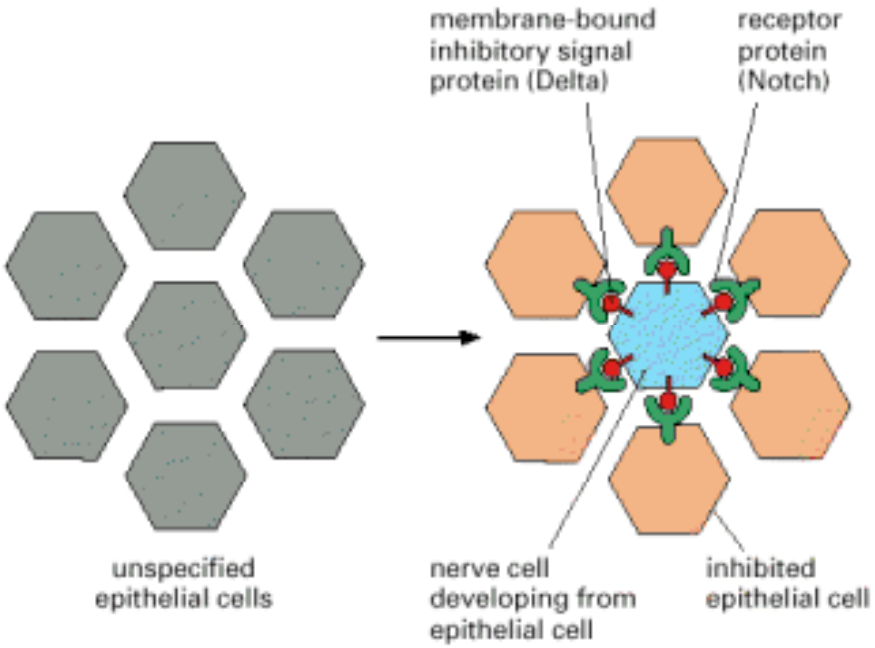
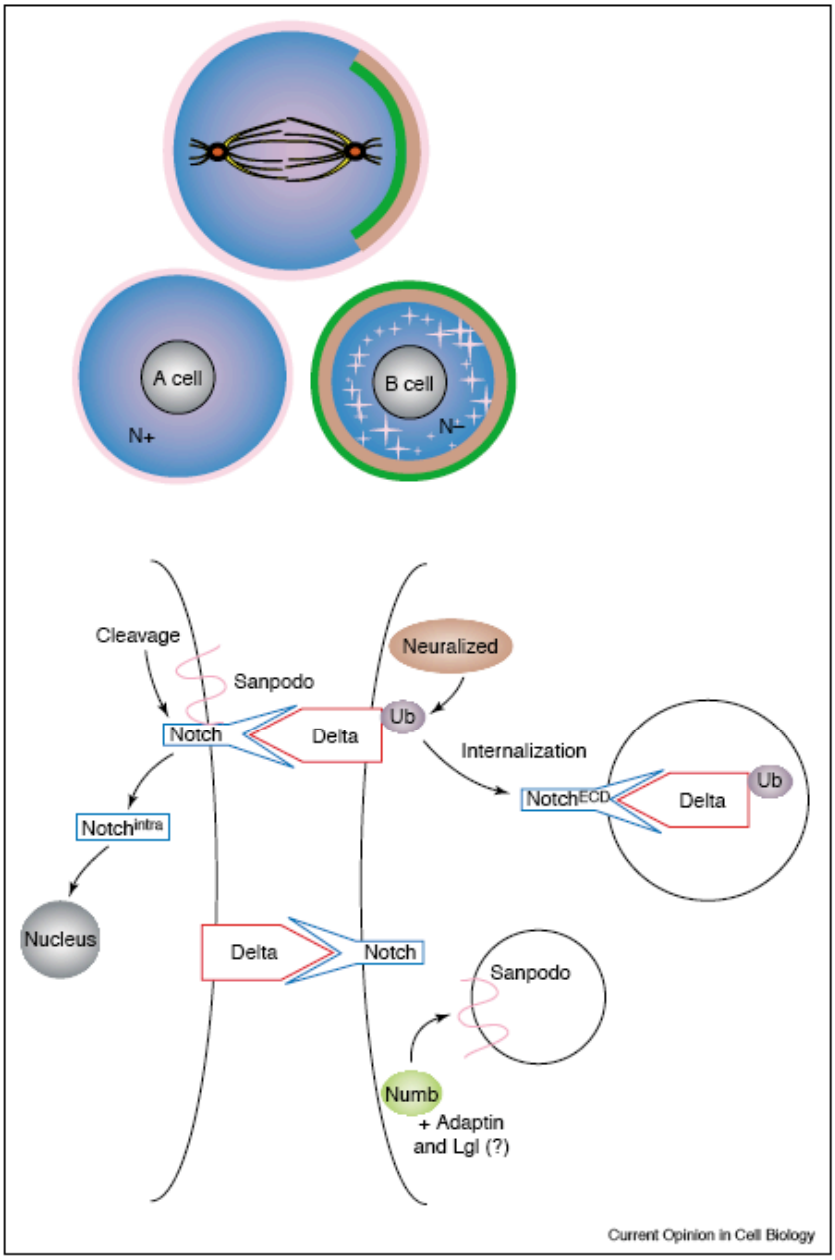


Figure 3 Lateral Inhibition

Initially all cells in the cluster have the potential to become a neuronal cell because all cells express the Delta ligand and Notch receptors. It is a matter of competition of which cell expresses active Delta. This cell will inhibit adjacent cells by the Notch pathway. This term of events is called lateral inhibition. (Alberts, 2002).



A possible model for cell fate determination during asymmetric cell division in *Drosophila* based on the actions of Numb (green), Neuralized (brown), Sanpodo (pink). In the metaphase precursor undergoing asymmetric cell division, Numb and Neuralized colocalize in a crescent, and Sanpodo is uniformly distributed in the membrane and in puncta in the cytoplasm (stars). After division is complete, Sanpodo is in the membrane in the A cell and Notch signaling (N+) is high. In the B cell Numb and Neuralized are cortical, Sanpodo is cytoplasmic and Notch signaling is low (N–). In the A cell Sanpodo interacts with Notch (coupled to mono-ubiquitinated (Ub) Delta) at the membrane and promotes cleavage of Notch to free the intracellular domain of Notch ($Notch^{intra}$) to signal in the nucleus. In the B cell, Numb (perhaps with α-Adaptin and Lgl) removes Sanpodo from the membrane, disrupting cleavage of Notch, while Neuralized mono-Ubiquinates Delta, which causes internalization of Delta-Ub and the Notch extracellular domain ($Notch^{ECD}$) to promote Notch signaling in the A cell. Empty circles represent putative vesicles.

Figure 4 Notch signaling pathway

This is a canonical pathway; one that is best known to represent the Notch pathway. (Roegiers and Jan, 2004).

C. Echinoid

Echinoid is a 1332 amino acid cell-adhesion transmembrane protein (TM) characterized by its N-terminal sequence and its IgC2-like domains. Ed has a 315 amino acids intracellular domain, which possibly functions in cell signaling. Loss of Ed results in an increase in neuroblasts (Ahmed et al 2003). It has been suggested that Echinoid's role is in the trafficking of the Notch ligand Delta by facilitating Delta endocytosis into endosomes (Rawlins et al, 2003). Ed also associates with Notch at the membrane as well as in vesicles of the cell. Furthermore Ed is co-localized in endosomal vesicles with the N^{ecd} and Delta during endocytosis (Rawlins et al, 2003). This specific arrangement suggests a functional link because Ed does not co-localize in the endosome with any other known cell-surface protein (Rawlins et al, 2003). Echinoid functions in taking away Delta and N^{ecd} from the cell surface after Notch activation. N^{icd} promotes epidermoblast formation (Louvi and Artavanis-Tsakonas 2006). This limiting potential allows the cell with the active Delta to become a neuroblast. Echinoid is expressed very dynamically during embryogenesis in the critical shaping of the CNS and PNS.

echinoid also functions as a negative regulator of the Epidermal Growth Factor (EGF) pathway. The EGF pathway is a signal transduction pathway, which has a critical role in a wide range of developmental processes such as changes in the cell shape and cytoskeleton. Loss of function mutations of echinoid shows extra wing and thoracic macrochaetae bristles, extra sensory organs, and improper eye formation. Ed loss-of-function results in hyperplasia of the nervous system, which is a very similar phenotype to Notch loss of function (Fig. 5) (Ahmed et al, 2003). Overexpression of Ed results in loss of many bristles and fewer SOPs. In addition, there is a reduced level of Delta ligand

at the membrane surface and vesicles, but no change in Notch protein level (N^{ecd} and N^{icd}) (Rawlins et al, 2003). This data suggests that Ed is more closely related to the trafficking/degradation for Delta than with Notch.

A vertebrate *echinoid* has not been identified yet, but it is known that the NGS is highly conserved in vertebrates. Due to the strong relationship between NGS and Echinoid it is very likely a human homolog of *echinoid* exists (Gridley, 1997). *echinoid* is also largely responsible for proper nervous system development, proper cell adhesion, and proper eye development. It is possible for these reasons that mutations of the vertebrate homolog of *echinoid* may result in similar diseases as mutations of NGS genes.

Earlier work has shown that Notch function is required for axonal outgrowth and synapse formation in invertebrate and vertebrate animals (Gall and Giniger, 2004). This study begins the analysis of the possible role of *echinoid* protein in these processes, specifically, its distribution in axons during their navigation. Furthermore, optimal conditions for studying Ed are presented. My observations have also led to the new finding of Echinoid localization during early stages of *Drosophila melanogaster* embryogenesis.

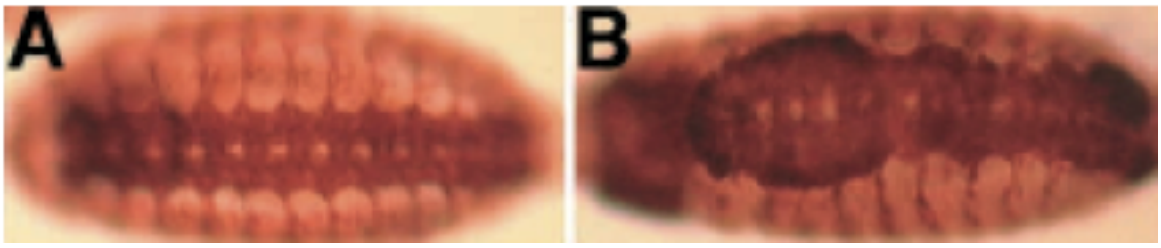


Figure 5 Neurogenic Ed mutant

anti-HRP-staining of the nervous system of **(A)** WT embryo and **(B)** homozygous Ed mutant embryo with hyperplasia of the CNS and PNS. (Ahmed et al, 2003).

III. Materials and Methods

W$^-$ Embryo Collections for Antibody Stainings

Embryos for *in-situ* fluorescent-antibody staining were collected either overnight or for two hours. Embryos were collected with distilled water. Chorions were removed using 50% bleach for five minutes or until white and shiny. The bleach was rinsed away. Embryos were places in a two-phase solution: lower phase of 3.7% formaldehyde and an upper phase of 50% Heptane. Embryos were shaken at 300rpm at room temperature (RT). Generally embryos were fixed for 20 minutes in Formaldehyde. For checking optimal conditions embryos were fixed for 10, 15, and 20 minutes. Two-hour embryos were fixed for 20 minutes. Formaldehyde was removed and Methanol was added at an equal volume of Heptane. Embryos were shaken for 1 minute, those without vitillin membranes sank into the methanol. The upper phase, Heptane, was discarded. Embryos were collected and washed 3 times in 100% Ethanol. Subsequently embryos were stored in 100% Ethanol at -20^{o}C.

W$^-$ Embryo Collections for Westerns

W- flies were put in collection bottles and starved for one hour at 25^{o}C. Later, one hour of heavy feeding on grape plates with a lot of yeast was done. Embryos were then collected future desired time span. Some 0:15-1:45h embryos were collected at 18^{o}C and used for initial Westerns. Embryos were collected with distilled water. Chorions were removed with 50% bleach for 5 minutes or until white and shiny. Bleach was washed with distilled water. Embryos were weighed before flash-freezing in liquid nitrogen. Embryos for Westerns were collected overnight and time collected at various ages at 25^{o}C. Stages include: **wide range:** 0-24h, **Stage 3-4**: 1:20-2:20h, **Stage 5**: 2:10h-2:50h, **Stage 6-11**: 2:50h-7:20h**, Stage 12-16**: 7:30h-16h. These timed embryos were used to detect Ed presence during development.

Immunohistology

Antibody stainings of embryos were done in two-day periods. Approximately 30 uL of embryos were used. Embryos were transferred from Ethanol to NaPBT. (10xNaPBS:

1.5M NaCl, 70 mM Na_2HPO_4 $2H_2O$, 30 mMNaH_2PO_4 $2H_2O$, pH 6.8) Dilute to 1xNaPBS and add 0.1% Triton-X100 to make 1x NaPBT, pH 7.4. Embryos were blocked for one hour in NaPBT/1% BSA at RT. Primary antibodies, Ed C5212 anti-Rabbit (RbAb) bleed #5 antibody and Ed α-P530 anti-Guinea Pig (GpAb), were used in 1:200 NaPBT/1%BSA overnight, 4°C. Next day, primary antibodies were washed with NaPBT, for 10,15,25,10 minute washes. Secondary antibody stains were done in dim light using FITC-conjugated anti-rabbit, FITC-conjugated anti-guinea pig, TR-conjugated anti-rabbit, TR-conjugated anti-Guinea Pig, or TR-conjugated anti-mouse 1:200 NaPBT/1% BSA one hour, room temperature. Secondary antibodies were washed with NaPBT, four 10,15,25,10 minute washes. Embryos were mounted on slides with fluorescent Vectashield mounting medium and viewed with confocal microscopy.

Double Stain

Embryos were simultaneously stained with two different primary antibodies generated from different animals systems. Two different fluorescent antibodies were used in overlap of images for confocal microscopy for comparison of known lineage markers and Echinoid.

Embryo Dissections

Some stage 13-17 embryos were dissected after antibody staining to expose the ventral cord for better viewing of axon outgrowth. Embryos were dissected using syringe needles along dorsal side. The gut was removed and epidermis opened to reveal ventral cord. Lineage markers such as Fascilin-2 and CC221 were used. Dissected embryos were mounted in Vectashield mounting medium and viewed using a confocal microscopy.

Protein Isolation

Approach 1: Using time-collected embryos, total embryonic proteins were fractionated for nuclear and cytoplasmic proteins. Place embryos in Homogenizing Buffer (Lysis Buffer (30mM Hepes pH8.0, 10mM KCl, 5mM $MgCl_2$, 0.5mM EGTA, 350mM Sucrose), 0.05mM phosphatase inhibitor, and Sigma protease inhibitor mix, 100mM DTT, and 200mM PMSF). Homogenized embryos sat in tritonX-100 for 30 minutes on ice. About

1/5 of the volume was aliquoted for total protein. The rest was spun at in SS34 rotor at 2911 rpm at 4^{o}C for 10 minutes. Cytoplasmic material stayed in the supernatant, which was aliquoted and frozen away. The pellet (nuclear protein) was resuspended and aliquoted in lysis buffer. Protein stored at -80^{o}C. (K.A. Bremer, personal communication).

Approach 2: Embryos were put in a pre-chilled glass tube in 3x volumes of 350-SHMDPSS Buffer (350mM sucrose, Hepes, $MgCl_2$, 1mM DTT, 2mM PMSF, 25ug mL^{-1} Spermidine, 25 ug mL^{-1} Spermine, additional 0.05mM phosphotase inhibitor, Sigma protease inhibitor mix, and Lysis Buffer). Homogenized with a glass pestle on ice for 30 strokes or until no intact tissue is seen. Later embryos had debri removed at 100g (800-1000rpm) for 1-5 min (all spins in Eppendorf centrifuge were done at 4^{o}C). Decant supernatant and let sit on ice for 30 minutes in 1% TritonX-100. Spin at 1000g (3,300rpm) for 15 min to get nuclei-I pellet. Re-spin the supernatant to get nuclei-II pellet; supernatant becomes cytoplasm-I. Re-homogenize pellets in 1-2x initial volume of 350- SHMDPSS and check under microscope for free-floating nuclei plus any intact cells. Centrifuge again at 800g (2,900rpm) for 10 min. Decant supernatant, cytoplasm-II (may be added to I or not). Re-suspend the pure nuclei in ½ initial volume in NE buffer (20mM Hepes, 1.5mM $MgCl_2$, 0.2mM EDTA, 20% Glycerol, 0.5M NaCl) and incubate with gentle rocking for 30 min at 4^{o}C (nuc*). Lyse the nuclei with an 18-gauge needle. Store cytoplasm and nuclei in aliquots, 3x100ul and 3x50ul for western blots at -80^{o}C. (Protocol modified from Cox, 2006).

Western Blot

Running Gel

10%, 5%, and 6% resolving gels (based on amount of acrylamide). Lower percent gels were used to observe better separation of high molecular bands. Gels were loaded with BioRAD broad range, high range, and precision markers (12-15uL). 4x Loading Buffer (250mM Tris HCl pH 6.8, 10%SDS, 5% β-Mercapto-EtOH, 50% Glycerol, 0.5% Bromophenoblue) was added to the thawed protein. Protein was immediately placed at 95^{o}C for 10 minutes and then centrifuged for a 2-3 minutes until ready to load. Between

15-18uL of protein was loaded into each well. Gels ran in Running Buffer (10xSDS running buffer: 30g/L Tris Base, 144g/L Glycine, 10g/L SDS) at 15mA until wells were stacked and protein passed into the resolving gel. The current was increased to 30-33mA and stopped when good separation of markers was observed. Electro-transfer of proteins from gel to nitrocellulose membrane occurred over night in Transfer Buffer (30g/L Tris base, 144g/L Glycine) at 4^{o}C in cold-water bath.

Ponceau S Staining membrane

Membranes were washed in 1xTBS (20mM Tris HCl pH 7.5, 150mM $NaCl_2$, pH 7.5)and stained to see if transfer worked using Ponceau S stain and subsequently washed in water until bands were clearly visible.

Western blot

Membranes were washed with 1xTBST (20mM Tris pH 7.5, 150mM NaCl, 0.5% Tween 20). Stripping Buffer (50mM Tris pH 6.8, 2% SDS, 100mM β-Mercapto-EtOH) was used when membranes had been used for a different antibody. and blocked in TBST/5% milk for 30 minutes at RT. Primary antibodies, Anti-Ed C5121 (1:2000) or Anti-Ed α-p530 (1:2000), were used in TBST/3%BSA overnight at 4^{o}C or 1 hour RT. Membranes were washed for three 10 minute washes in TBST. Secondary antibodies Rabbit-anti-HRP (1:10000, Jackson Laboratories) and Guinea Pig-anti-HRP (1:15000, Jackson Laboratories) were used in TBST/5% milk for one hour at RT. Membranes were washed for three 10 minute washes in TBST and later kept in TBS until ready to develop.

Developing film

The membranes were places in 2mL of non-sensitive color reaction reagent and 2mL of sensitive color reaction reagent for one minute. Initial one minute exposure to film was done to assess signal as well as strength of signal. Various timed exposures were done to get optimal banding seen without background.

V. Results

A. Echinoid in axon formation

In later stages of embryonic development Ed is apparent in the CNS and during axon formation. Two different anti-Ed antibodies were used for in situ embryo stains. One was RbAb c5212, which was raised to the N-terminus of Ed. The other was GpAb α-p530, which was raised to the C-terminus of Ed (Ahmed et al, 2003).

Pictures using confocal microscopy have shown that Ed is more concentrated at the commisures of the CNS. Furthermore a spotted look along the ventral cord is evident (Fig. 6). At a higher magnification the spotted look is more apparent throughout the CNS. The high density of Ed in specific locations is somewhat reminiscent of Rawlins' study of Ed in cell culture (Rawlins et al, 2003). In dissected wild type embryo, Ed is clearly seen along the proximal lengths of the axons extending from the ventral cord (Fig. 6). The middle of axons becomes fainter, but it Ed is clearly at the distant end of the axons. Using the C-terminus anti-Ed, axon formation can be seen more easily (Fig. 7). During axonal navigation Ed can be seen at the ends of axons where growth cones are located (Fig. 6). Growth cones use cues from outlying structures to determine their path (Sanes et al, 2006). Pictures show axons navigating from the CNS to the periphery where trachea are forming (Fig 7). Fasciculation of axon and trachea appear to take place.

Ed's presence during axonal outgrowth triggered our study to use double stains of known cell lineage markers to determine whether we can identify the Ed positive neurons. These antibodies mark defined subsets of neurons in the nervous system. Double stains show that Ed does not seem to overlap with 22c10 or Fasiclin-2 (Fig 8-10). This shows that Ed is most likely present in a different subset of neurons, but it is possible that

the antibodies were very sensitive to the double stain and could not show accurate overlap. Additional known markers should be used in the future to identify the Ed positive lineages.

Reproducible immuno-histological assays show that Ed is found in axonal outgrowth. Since it is located at the growth cones it is possible that Ed has a role in axonal navigation. In addition, Ed was seen in tracheal pits and developing trachea. A close proximity of trachea and Ed positive axons is evident. Axon navigation relies heavily on trachea. Ed is seen in both areas, which further implicates its role in axonal outgrowth and navigation. Identifying which neurons play in the role of Ed requires additional observation with known subset markers. Knowing the neurons will help to find which structures Ed neurons innervate.

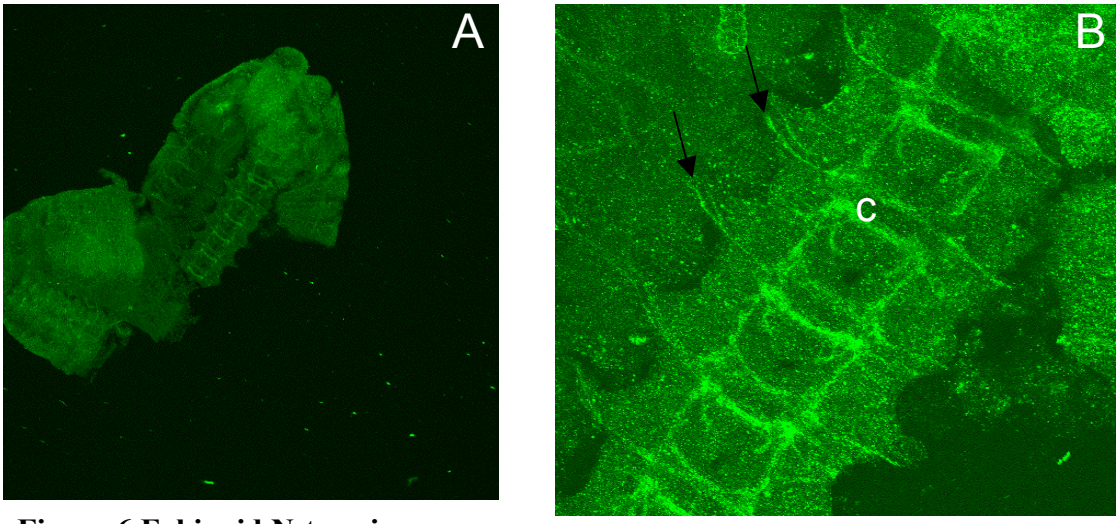


Figure 6 Echinoid N-terminus

(A) WT dissected embryo. FITC-RbAb anti-Ed c5212. Shows ventral cord and beginning axon formation. **(B)** higher magnification shows more detailed expression of Ed in commisures (c) and in growth cones (arrows)**.**

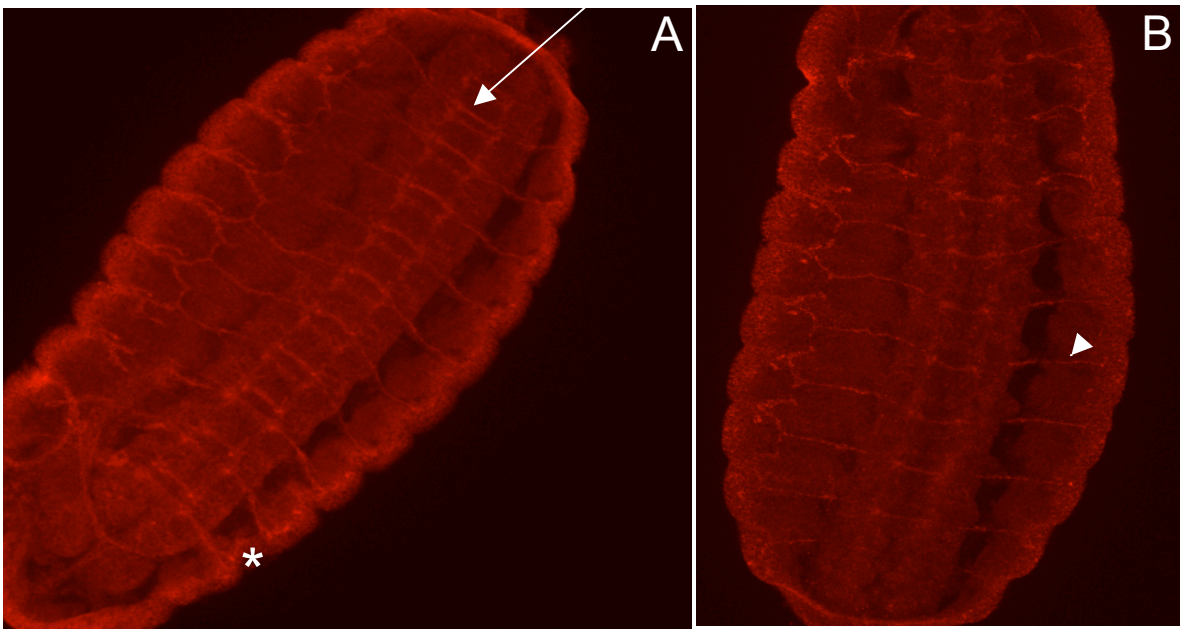


Figure 7 Echinoid C-terminus

Whole WT embryo. Primary Ed antibody: TR anti-Ed α-p530 anti-Guinea pig. 10 min fixation. Embryos show axon formation and Ed presence in CNS, commisures (arrow), axons (arrow head), and growth cones along epidermis. Axons extend towards developing trachea (star).

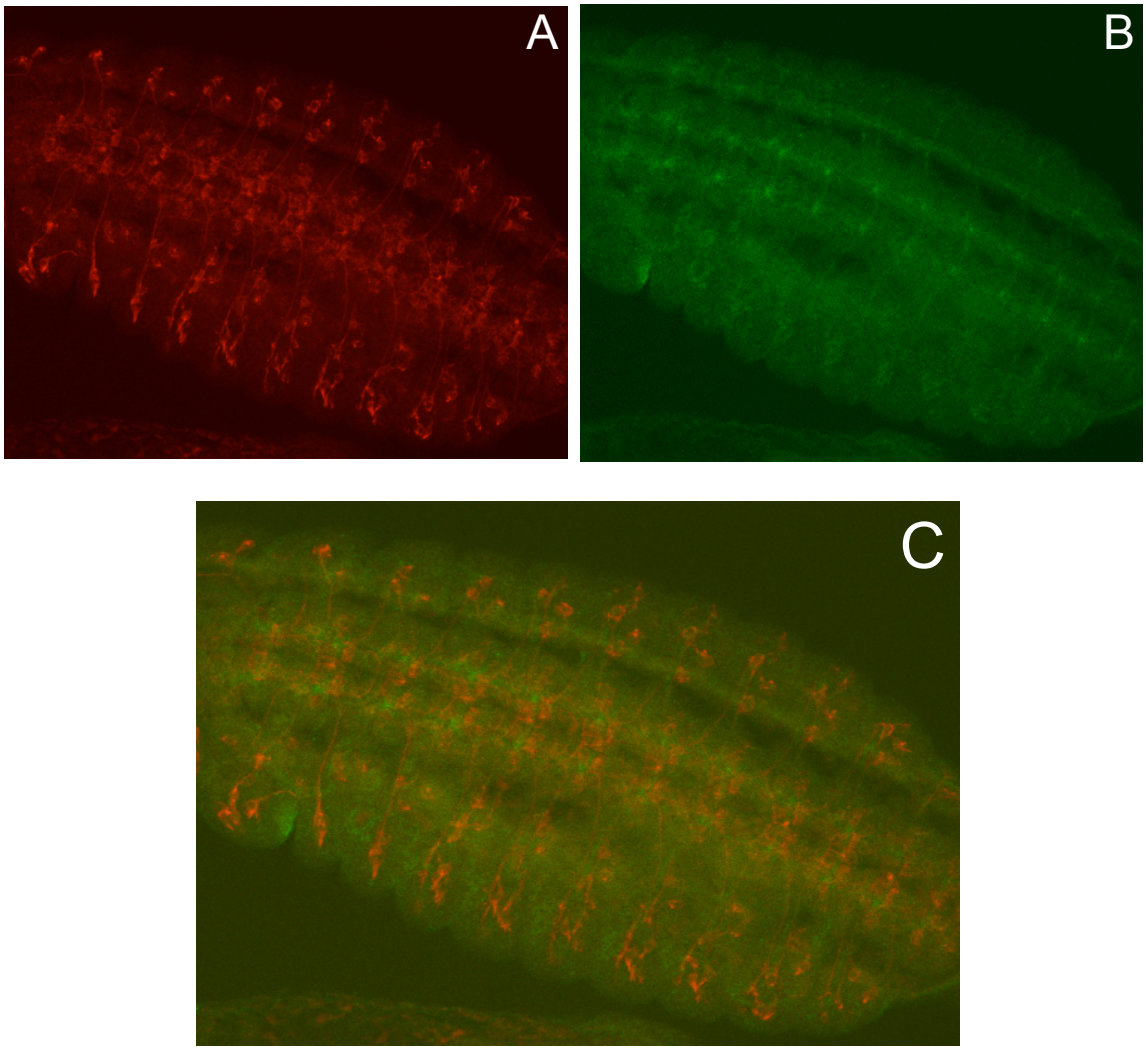


Figure 8 Ed and 22c10

WT whole embryo showing ventral cord. **(A)** TR-monoclonal MAb 22c10 (red). **(B)** FITC-RbAb anti-Ed c5212 (green). **(C)** double stain of Ed and 22c10 shows comparison lineage. Ed does not seem to overlap with 22c10 lineage subset marker at axons or CNS (arrow and circle).

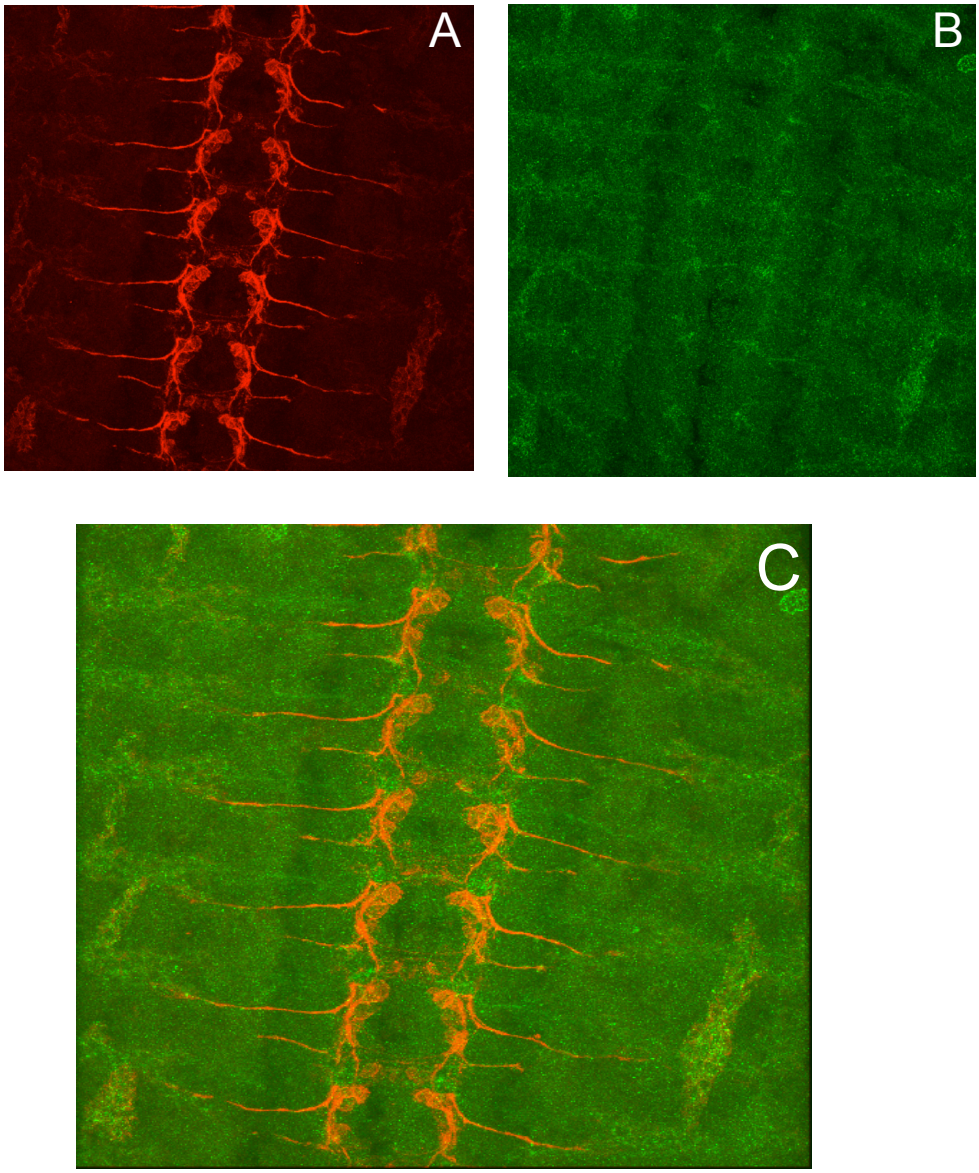


Figure 9 Ed and 22c10 dissected

WT dissected embryo**.** Shows a high-resolution view of axon extension. **(A)** TR-monoclonal MAb 22c10 (red). **(B)** FITC-RbAb anti-Ed c5212 (green). **(C)** double stain of Ed and 22c10.

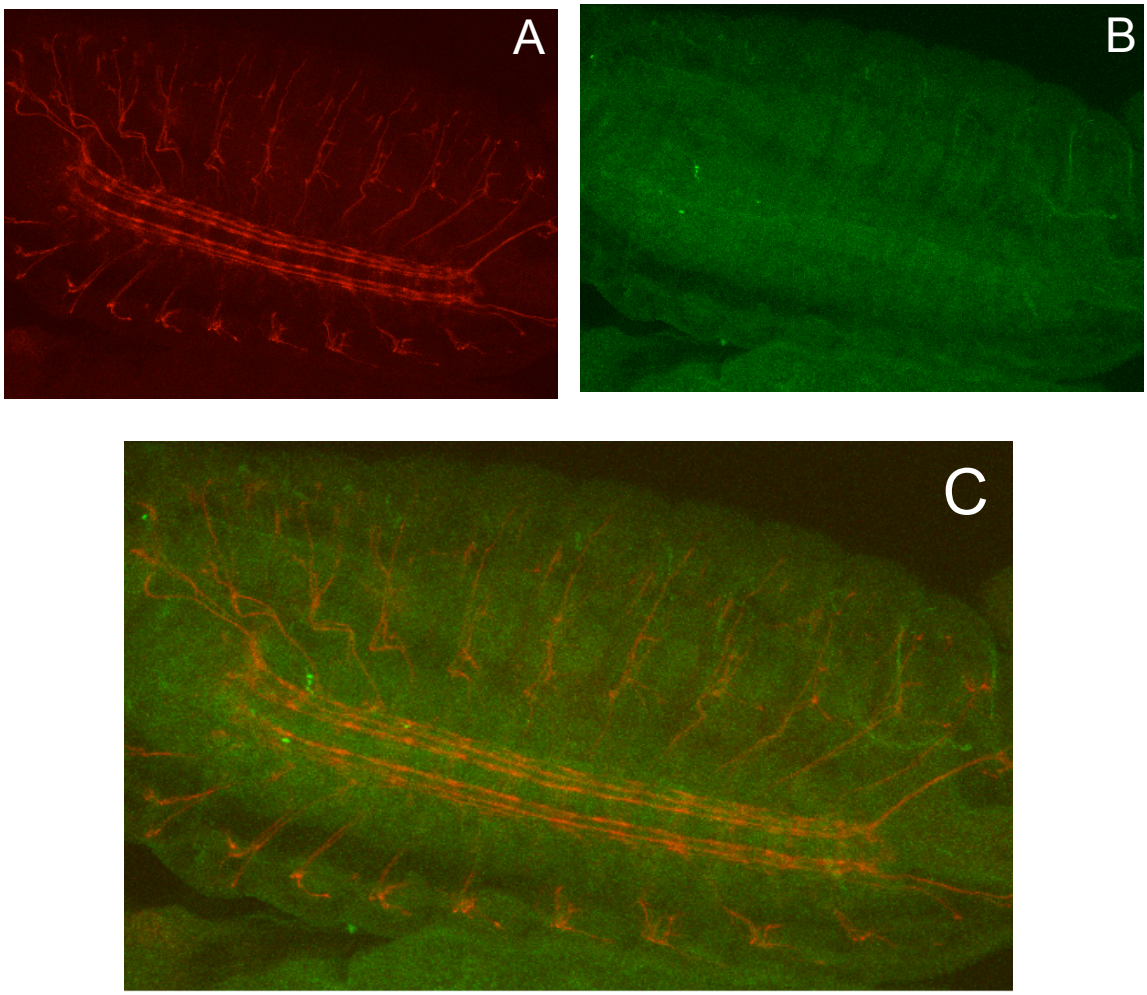


Figure 10 Ed and Fasiclin-2

WT whole mount embryo **(A)** TR-MAb Fasiclin-2 (red). **(B)** FITC-RbAb anti-Ed c5212 (green). **(C)** double stain of Ed and Fasiclin-2 shows comparison lineage. Ed does not seem to overlap with Fasiclin-2 lineage subset marker at axons or in CNS (arrows).

B. Optimal conditions

During the course of this work it became apparent that fixation conditions used may not have been optimal for the detection of Ed in axon outgrowth. To determine whether more optimal conditions could be established part of my work was to find the optimal conditions for immuno-histological experiments. A critical variable was in the fixation time during embryo preparations. Three different times were used: 10, 15, and 20 minutes. For viewing axonal outgrowth the best time for fixing the embryos was 10 minutes (Fig. 11). Anything less than 10 minutes causes the morphology to suffer substantially. However the short time causes problems with double stains (data not shown). Even with the optimal conditions found, the anti-Ed antibodies could still be very sensitive with other antibodies in the double stain figures shown. 10 minutes provided the best fix for the antibody to get into the embryo as well as keep its shape throughout the experiment. We found that the 15 minute fixation times can possibly work in the future for secondary markers e.g. growth cones. For nuclear localization (see below) of Ed, 20 minute fixation times worked the best (Fig.17).

In a study done by Lapante, Echinoid was also found to play a role in epithelial morphogenesis, specifically contractile actomyosin, which may explain why Ed mutant embryos were too brittle to get a clean staining and to dissect (Lapante 2006). Temperature-sensitive Ed mutant immuno-histological stainings were done, but optimal conditions were not found and no data collected. During dissections embryos completely fell apart and proper visualization of the nervous system was not possible (data not shown). Future work will have to determine optimal conditions for temperature sensitive

Ed mutants in order to study the role of Ed and its potential mechanism in axon growth and nuclei localization.

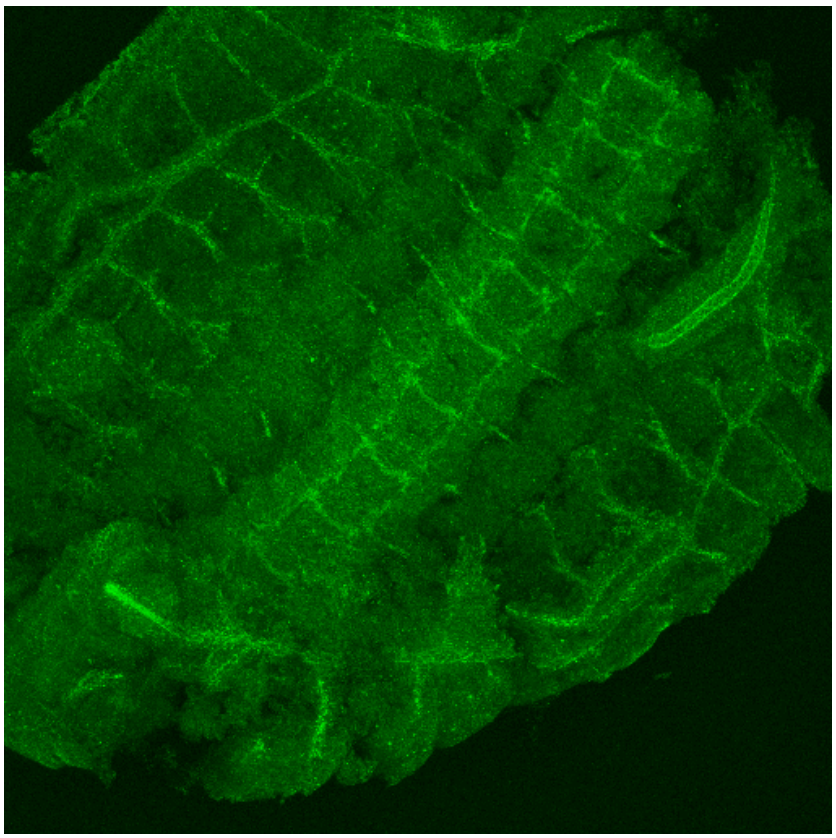


Figure 11 Optimal Condition for Axons

WT dissected embryo. FITC-RbAb anti-Ed c5212. 10 minute fixation represents optimal condition for best visualization of antibody stain while still allowing for reasonable dissections.

C. Protein Localization

An unexpected observation was made in the course of this study. After viewing antibody stainings of Echinoid in 0-24 hour old W^- embryos for axonal outgrowth, signal was seen in the nuclei of early syncytial embryos (0-2 hour old). The embryos were in stage 3-4 of development when it is essentially a giant cell with nuclei. In early embryos only low localization of Ed is evident (Fig.12). The next stage shows some signal, which indicates presence of Ed (Fig. 13). Early before outset of pole cell formation, signal starts to be evident in the nuclei of the embryo (Fig. 14). During pole cell formation this signal becomes more pronounced (Fig. 15). Shortly after pole cell formation the nuclear signal in the embryo is no longer evident, but remains visible for a short period in the new pole cells (Fig. 16). During onset of cellularization no nuclear signal is evident and the newly forming cell walls are labeled (Fig. 17).

The surprising observation led us to ask whether Ed could indeed be shown to localize with nuclei. In a first experiment we isolate proteins from 0-24 hour old embryos. Proteins were isolated into a cytoplasmic fraction and a nuclear fraction using predetermined centrifuge speeds and times. The proteins were loaded into a gel to be used for western blots. The same Ed antibodies were used to visualize whether Ed is present. Ed has a calculated molecular weight of 153 kDa without modifications. There was a difference in the banding between cytoplasmic and nuclear fractions of 0-24 hour embryos; a clear band was seen in the nuclear fraction between the 250 kDa and 150 kDa markers (Fig. 18). This band is detected with both anti-Ed antibodies; α-p530 are detecting the C-terminus and c5212 are directed against the N-terminus.

To get additional proof, a timed collection of embryos was made. The embryos were collected for two hours so that the developmental range was between zero and two hours. They were homogenized using a previously used protein isolation protocol. The same anti-Ed antibodies, c5212 and α-p530, were used on western blots. The blots showed a stray band for nuclear fractions and only a weak band in the cytoplasmic fraction (Fig. 19). Various stages of embryos were also collected and homogenized using a more stringent protocol to obtain better purified nuclear fraction (Cox and Emili 2006).The stages are stage 3-4, stage 5, stage 6-11, and stage 12-16. This experiment was performed to determine whether Ed is localized not only in the nuclei of the syncytial embryo, but also possibly during later stages (Fig. 20). Only a weak band is seen in the cytoplasm, but a distinct band is evident in the nuclear fraction. Both anti-Ed antibodies produce the same signal for this band. As mentioned earlier, Ed is seen in the cell membrane after cellularization (stage 5). Surprisingly during later stages little signal is detected in the cytoplasmic lane despite strong cell membrane signal in *in situ* antibody reactions (Fig. 20). The cause for this is not clear at present, but it is likely the membrane fraction was lost during protein isolation. More work is necessary to confirm this observation. In addition, control will be necessary to determine the purity of the isolated nuclear fractions.

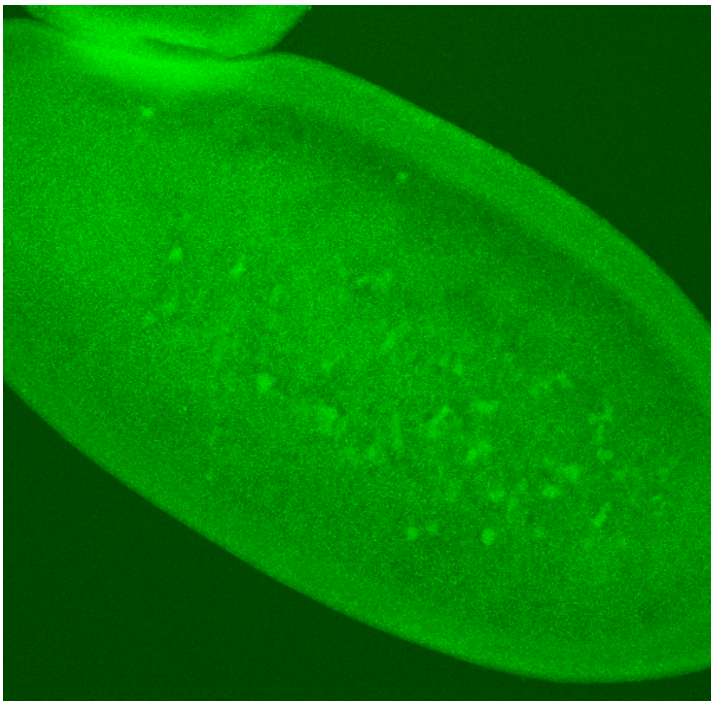


Figure 12 Earliest Stage

WT whole mount embryo. 20 minute fixation. FITC-RbAb anti-Ed c5212. Early embryo. Weak signal is detected.

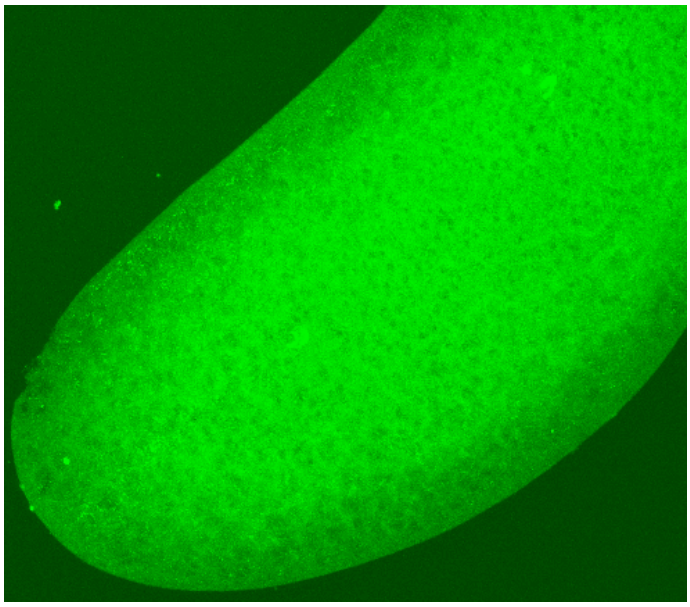


Figure 13 Initial Ed translation

WT whole mount embryo. 20 minute fixation. FITC-RbAb anti-Ed c5212. Signal readily detected, but no distinct organization of labeling is evident.

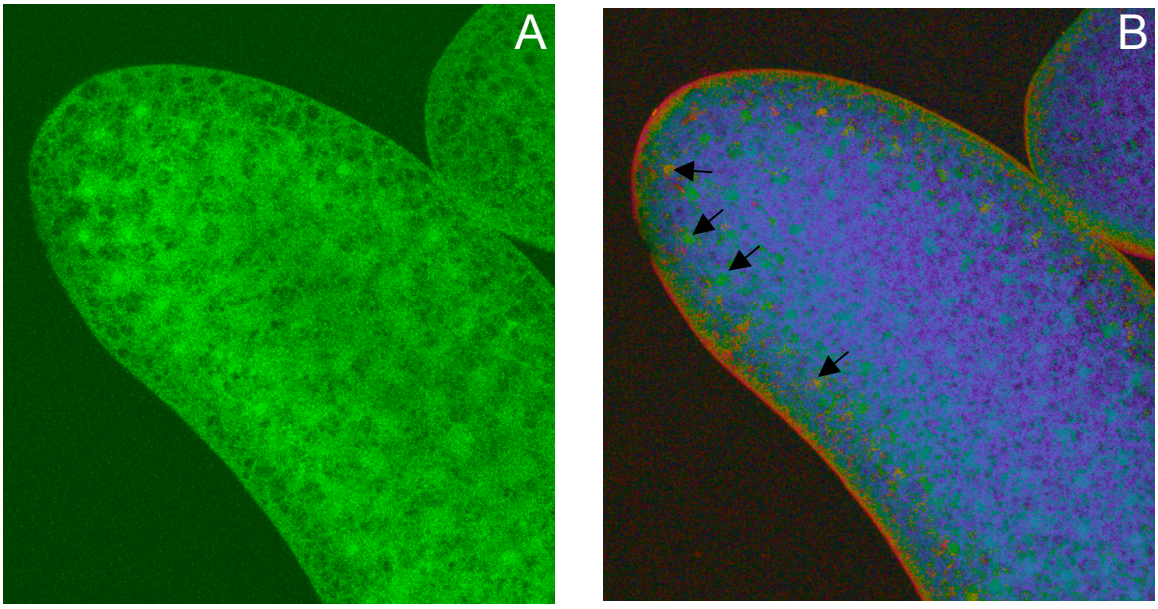


Figure 14 Start of nuclear localization

WT whole mount embryo. 20 minute fixation. FITC-RbAb anti-Ed c5212. **(A)** early embryo at start of Ed nuclear localization. **(B)** intensity picture to show density of Ed in nuclei (arrows).

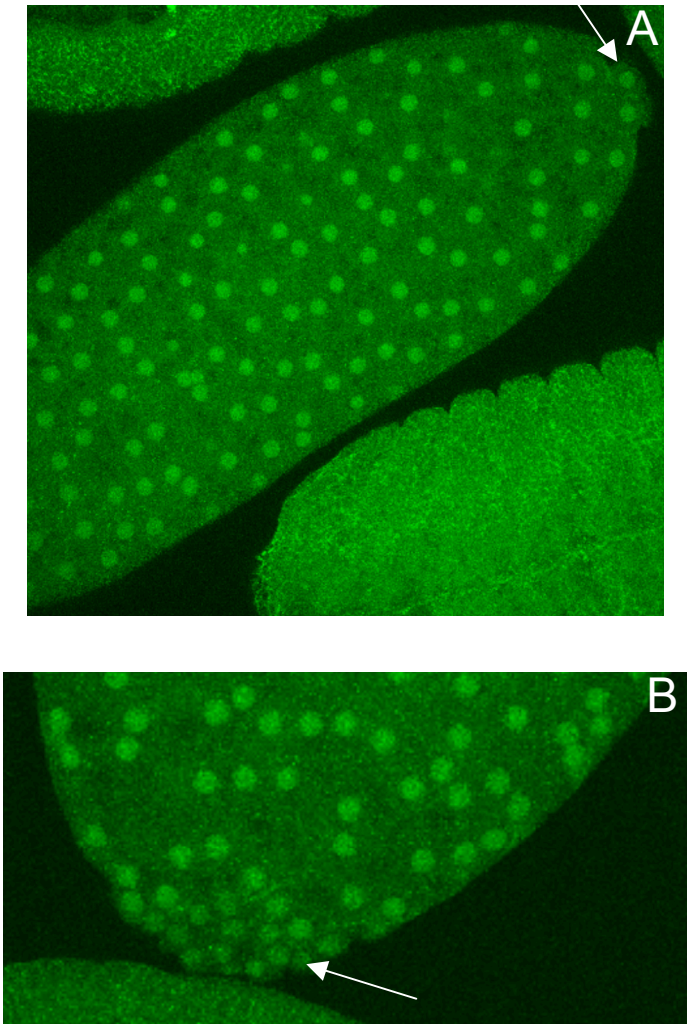


Figure 15 Pole cell formation

Whole WT embryo. 20 minute fixation. Primary Ed antibody: RbAb anti-Ed c5212. **(A)** embryo during pole cell formation shows Echinoid localized in or near nuclei **(B)** and in pole cells (arrows)

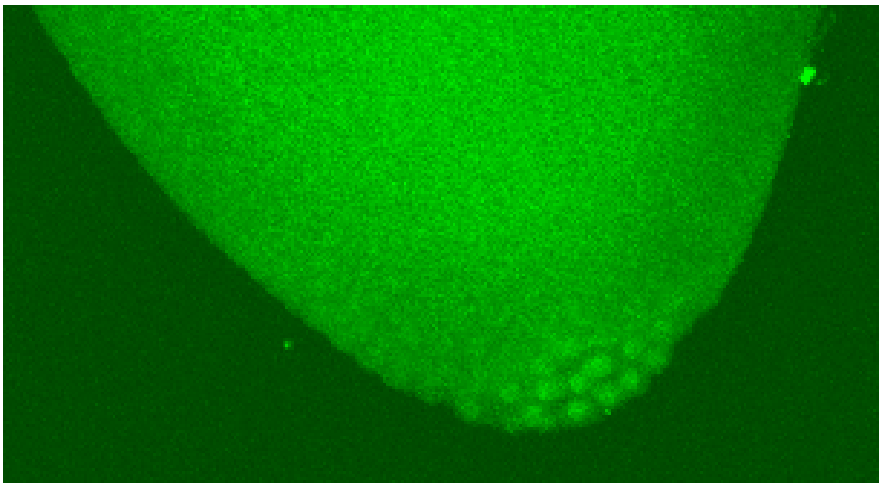


Figure 16 Lingering Ed in Pole Cells

FITC-RbAb anti-Ed c5212. Ed protein still seen in pole cell nuclei for a short period after pole cell formation.

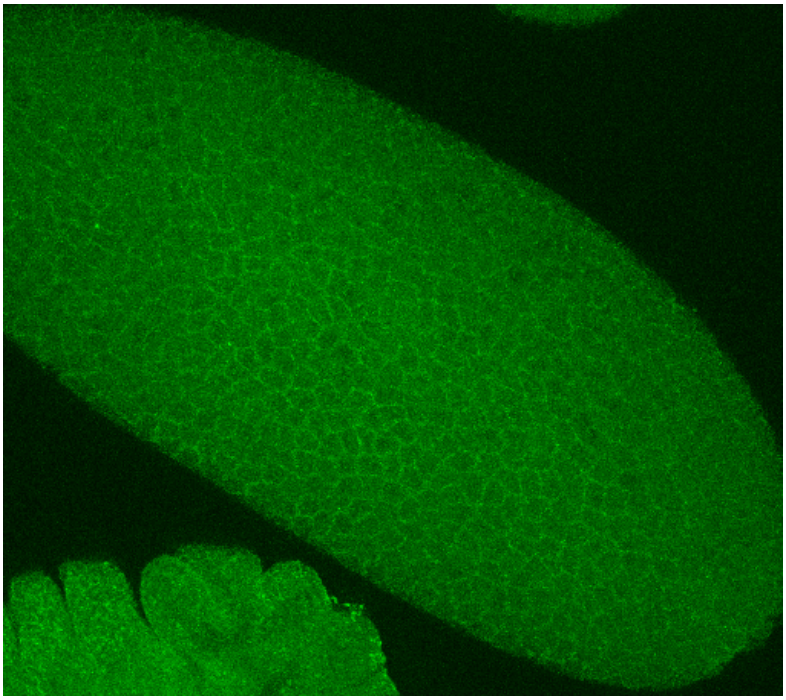
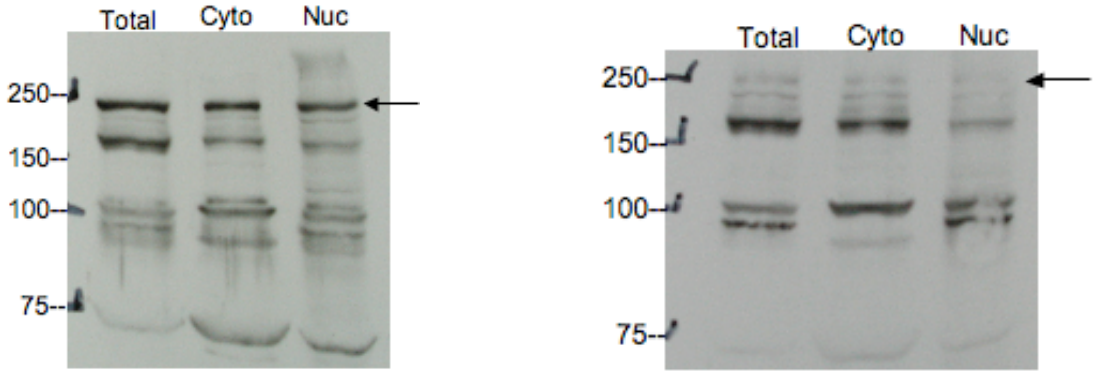


Figure 17 Blastoderm stage

FITC-RbAb anti-Ed c5212. Blastoderm embryo during onset of cellularization. Echinoid at cell membrane.

A

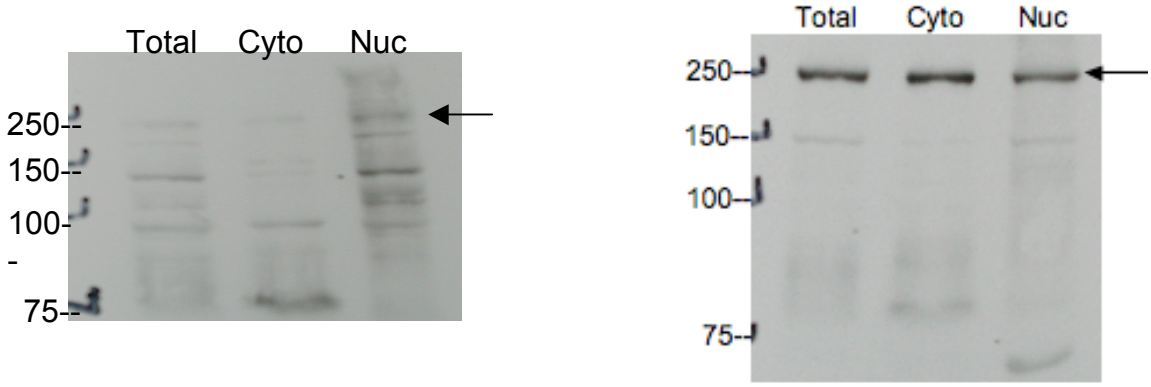
anti-Ed RbAb c5212



Membrane A **Membrane B**

B

anti-Ed GpAb α-p530



Membrane A **Membrane B**

Figure 18 Western 0-24h embryos

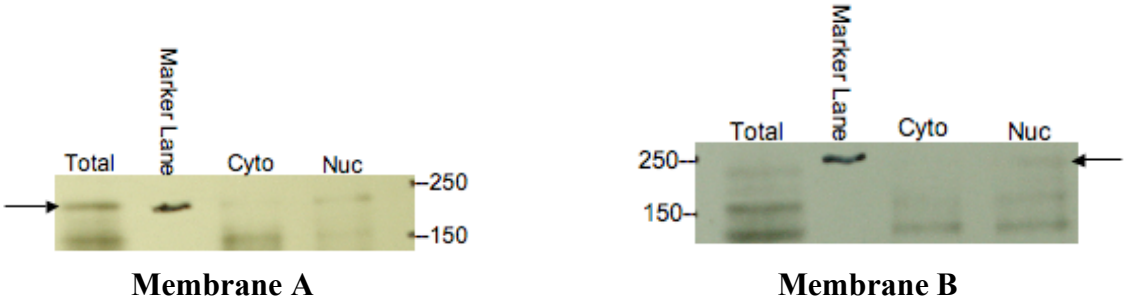
WT 0-24 hour old embryos. Initial protein isolation shows a difference in banding between nuclear and cytoplasmic fractions of Ed (arrows).

(A) Membrane A and B probed with anti-Ed c5212 (Rabbit)

(B) Membrane A and B probed with anti-Ed α-p530 (Guinea Pig)

A

anti-Ed RbAb c5212



B

anti-Ed GpAb α-p530

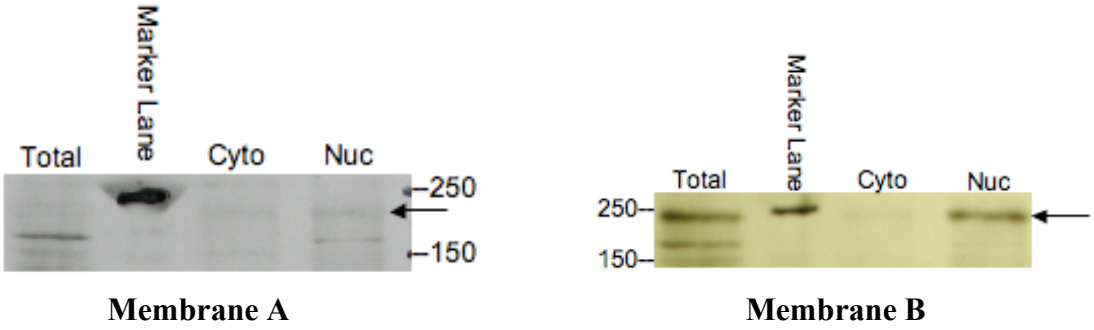


Figure 19 Western 0-2h embryos

WT 0-2 hour old embryos. Embryos show nuclear localization of Ed during syncytial pre-blastoderm stages. Ed bands are marked with arrows.

(A) Membrane A and B probed with anti-Ed c5212 (Rabbit)

(B) Membrane A and B probed with anti-Ed α-p530 (Guinea Pig)

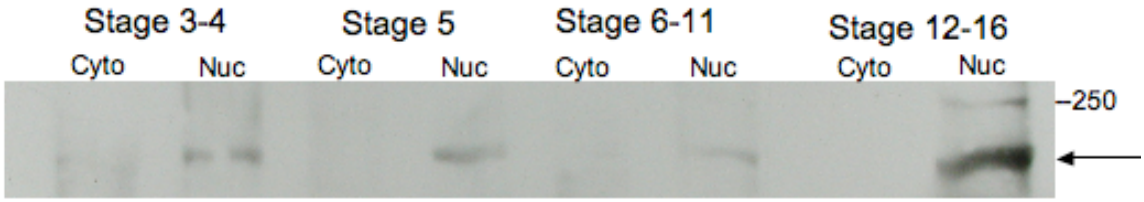


Figure 20 Western blot analyses of staged embryos

A faint band was detected using RbAb anti-Ed c5212. Ed band is located between 250 kDa and 150 kDa (arrow). There is a distinct band in the nuclear fractions, while weak bands are visible in the cytoplasmic fraction.

VI. Discussion

Embryonic phenotype in Ed mutants revealed hyperplasia of the central nervous similar to loss-of-function of Notch (Ahmed et al 2003). Additionally Ed mutants also show a loss in epidermis that is also seen in the NGS mutants. The NGS has also been found to control neuronal cell fate during PNS development. Initially this studied sought to find Echinoid's involvement in axonal outgrowth. While Ed was present during axonal navigation another observation was made that required further analysis. After viewing antibody stains of later staged embryos in an overnight collection, the younger embryos seemed to show localization of Ed in nuclei during syncytial stages. This spotted effect was visualized in many stains and so the localization of Ed became of interest. The N^{icd} acts as a co-factor and binds to proteins in the nucleus to form transcription factors. Echinoid's localization may have a similar role in gene control in early embryo development.

One question we sought to answer was that after our first Western of the 0-24hour embryos was whether the banding pattern was an artifact? Results show that this is not the case because of three substantial reasons. First, it wasn't the secondary antibody because the band did not show when the pre-bleed primary antibody was used. Also when the membranes were switched with antibodies, the same banding pattern appeared. Second, nuclear extracts show that Ed is present with higher density in the nuclear fractions in the early embryos too, when Ed is first seen. Third, the same banding patterns are seen with both C-terminus and N-terminus antibodies of Ed.

The last observation led us to another question of whether nuclear Ed is a full-length receptor protein that is being endocytosed or a partial protein? Comparing the two

antibodies, the same full-length band is seen with both the C-terminus and the N-terminus antibodies of Ed. Both antibodies bind to apparently this same band on the blot. This distinct localization of Ed at a specific stage of development may point to a role of Ed as a cell-surface receptor that undergoes nuclear translocation with possible nuclear signal transducing functions. Several research papers have been published about cell surface transmembrane proteins that are trafficked to the nucleus in full-length and may act in transcriptional regulation. These were seen with growth factor receptors of FGF and HB-EGF that are targeted to the nucleus (Bryant 2005 and Hieda 2008). It is possible that the entire transmembrane receptor is involved in nuclear-trafficking as well as potentially at later stages. Future work should attempt to find a possible nuclear localization signal in the Echinoid protein. An experiment that can also be done would be to create GFP-tagged fusion proteins of Ed one at the C-terminus and another at the N-terminus to see whether Ed is cleaved or if it is endocytosed as a full-length protein.

During the early stages of embryonic development (stage 1-4), most of the proteins present are either supplied by the mother or are translated from maternal mRNA. The embryo has not yet begun zygotic transcription until after cellularization. Is early stage Echinoid protein translated from maternal mRNA? This question needs a lot of background information before it can be answered, but one aspect to look at would be Ed's role in the nuclei. In addition a double stain with Ed and DAPI should be done to show that Ed is in fact located in the nuclei. Ed was also seen in pole cells during syncytial blastoderm stage and even after cellularlization. Ed's role in pole cell formation is also a new field of study. In addition, the observation of Ed in spots during axonal outgrowth and in syncytial may indicate localization to endosomes. Future experiments

include double labeling endosomal markers such as ubiquitin with Ed. Co-localization experiments with Delta and Notch should also be done.

The Western blot of staged embryos provided numerous problems at the detection step. Most times bands would not be seen at all due to old kits, wrong dilutions of new kits, old antibodies, ECL substrate blow-out, etc. At times some lower bands would show, but no upper bands. Currently we are redoing the Western blot for the four stages of embryos to recount for potential loading and protein stability differences. It is also possible that bigger proteins such as Ed degraded between thawing and loading of the gel. Proteins are stored with protease inhibitor, but it is possible that storing proteins at -80^{o}C can still result in degradation due to subsequent thawing.

VI. Outlook

My work raises two issues. Now that it is known Ed is present in axonal outgrowth, what is its role in development of the subset of neurons? What phenotypic changes occur in temperature-sensitive Ed mutants? What are the best conditions for immuno-histological assays on Ed mutants considering epidermal loss? The other issue opens a whole new field within this topic of Echinoid function. What is the potential mechanism of Ed in early embryo development? Does it affect pole cell development or morphogenesis? Is early Ed a maternal mRNA? If so, what role does it have in the nucleus?

VII. Acknowledgements

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VII. References

Ahmed A, Chandra S, Magarinos M, et al. (2003) echinoid mutants exhibit neurogenic phenotypes and show synergistic interactions with the Notch signaling pathway. Development 130 (25): 6295-6304

Alberts, Bruce; Johnson, Alexander; Lewis, Julian; Raff, Martin; Roberts, Keith; Walter, Peter. Molecular Biology of the Cell. 4th ed. New York and London: Garland Science; c2002. Figure 15-70.

Brody, Thomas Ph.D. "The Interactive Fly". 1995. http://www.sdbonline.org/fly/aimain/1aahome.htm

Bryant, David M. and Stow, Jennifer L. (2005) Nuclear Translocation of Cell-Surface Receptors: Lessons from Fibroblast Growth Factor. Traffic. 6: 947-954

Campos-Ortega, José A. and Hartenstein, Volker. The Embryonic Development of *Drosophila melanogaster*. Springer-Verlag. Berlin Heidelberg. 1985. 20-84.

Cox, Brian and Emili, Andrew. (2006) Tissue subcellular fractionation and protein extraction for use in mass-spectrometry-based proteomics. Nature protocols.1 (4): 1872-1878

Gall, Maude Le and Giniger, Edward. (2004). Identification of Two Binding Regions for the Suppressor of Hairless Protein within Intracellular Domain of *Drosophila* Notch. Journal of Biological Chemistry. 279 (28): 29418-29426.

Gridley, T. (1997). Notch Signaling in Vertebrate Development and Disease. Molecular and Cellular Neuroscience. 9. 103-108.

Hieda, M. et. al. (2008) Membrane-anchored growth factor, HB-EGF, on the cell surface targeted to the inner nuclear membrane. The Journal Cell Biology. 180 (4): 763-769.

Hortsch, M. (2003) Drosophila Echinoid is an antagonist of Egfr signalling, but is not a member of the L1-type family of cell adhesion molecules. Development 130 (22): 5295-5295

Laplante, Caroline, Nilson, LA, et al. (2006) Differential expression of the adhesion molecule Echinoid drives epithelial morphogenesis in Drosophila. Development 133 (16): 3255-3264

Louvi, Angeliki and Artavanis-Tsakonas, Spyros. (2006) Notch signaling in vertebrate neural Development. Neuroscience. Vol. 7.

Rawlins, Emma L., Lovegrove, Bridget, and Jarman, Andrew P. (2003) Echinoid facilitates Notch pathway signaling during Drosophila neurogenesis through functional interaction with Delta. 6475-6484.

Sanes, D., Reh, T., Harris, W. Development of the Nervous System. 2nd ed. New York: Elsevier Inc. 2006.